Study code: 3000-4203
(Old code 1903003)

Study title:

Effects on spontaneous motor activity of mice. Hydroxymatairesinol.

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Study Report

EFFECTS ON SPONTANEOUS MOTOR ACTIVITY OF MICE

HYDROXYMATAIRESINOL

Study number: **P11.6-1999**

Date: 20.8.2002 (version 2)

Hormos Medical Ltd.
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FINLAND

Sponsor Study number: 1903003

CONFIDENTIAL

PreFa
Preclinical Pharmacology Research Unit
University of Turku

Key Words: Hydroxymatairesinol (HMR), safety pharmacology, locomotor activity

1. GENERAL

1.1. SIGNATURES

Title Effects on spontaneous motor activity of mice; Hydroxymatairesinol

PreFa study number: P11.6-1999

Sponsor study number: 1903003

Test item: Hydroxymatairesinol (HMR)

This Report version 2 replaces the 1st version dated 14.6.2000. Following change has been made:

1. **Section 2.3.3. Rationale for dose selection:** Reference to a study demonstrating the antitumor activity of HMR has been added.

This report is a complete and accurate account of the methods employed and the data obtained

20.8.2002

Aapo Honkanen
Study Director

date

1.2. TABLE CONTENTS

1.3. PURPOSE OF THE STUDY

The purpose of this study was to assess safety pharmacological properties of the hydroxymatairesinol (HMR) by assessing its effect on spontaneous motor activity of mice. In addition to HMR, the effects of another compound, HTS-101 were tested in the same experiment. Same control group (vehicle treatment) and reference compound-treated groups were used in the evaluation of the effects of these compounds. The results from HMR and HTS are reported separately.

1.4. SUMMARY

The spontaneous motor activity of the animals (horizontal and vertical) was measured in transparent polypropylene cages with Photobeam Activity System (PAS, Cage Rack®, San Diego Instruments, San Diego, USA). Ambulatory, vertical and fine movement activity counts accumulated over measurement period were recorded. Vehicle (PEG 300), HMR (10, 30 or 100 mg/kg) or reference compound, amphetamine (2 mg/kg), were given p.o. (Experiment 1) or s.c. (Experiment 2), while another reference compound, medetomidine (30 µg/kg), was given s.c. Immediately after the treatments, the animals were placed in the activity measurement cages and their activities were recorded for 180 min.

In the Experiment 1, only medetomidine significantly decreased the motor activity of the animals during the first 90-min period of the test, while HMR and amphetamine were without effect. In order to demonstrate locomotor stimulation, an additional experiment was performed (Experiment 2), in which amphetamine (2 mg/kg) was given subcutaneously. The effect of a largest HMR dose (100 mg/kg, p.o.) was tested as well. Again, HMR did not alter any of the measures of motor activity when compared to the control group. In contrast, amphetamine induced significant motor stimulation. These results demonstrate that HMR (10-100 mg/kg, p.o.) does not alter the motor activity of the NMRI mice.

1.5. GUIDELINES

The study procedures described were based on the guidelines listed below:

- Asetus Kokeellisiin ja muihin tieteellisiin tarkoituksiin käytettävien selkärankaisten eläinten suojelemiseksi tehdyn eurooppalaisen yleissopimuksen voimaansaattamisesta. Suomen säädöskokoelma n:o 1360/90. Helsinki, 21 joulukuuta 1990
- European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, European Treaty Series No. 123, (EU n:o 609/86) (Official Journal of the European Communities No L 358) Strasbourg 24th November 1986.

1.6. APPROVAL FROM THE ANIMAL CARE AND USE COMMITTEE

The study has a permission from the animal care and use committee of University of Turku n:o 922/99.

1.7. SPONSOR

Hormos Medical Ltd.
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1.8. RESEARCH LABORATORIES

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1.9. STUDY DIRECTOR

Aapo Honkanen M.Sc. (Pharm.), Project Manager

1.10. PERSONNEL INVOLVED IN THE STUDY

PreFa/Department of Pharmacology and Clinical Pharmacology
Esa Korpi, MD, Ph.D. Professor of Pharmacology
Aapo Honkanen, Project Manger
Elisa Riuttala, Laboratory Technician

CRST(Clinical Research Services Turku)/Biostastics
Esa Wallius

1.11. TIME TABLE

Start of animal acclimatization:	1.9.1999 and 5.1.2000
Experimental starting date:	7.10.1999
Experimental completion date:	14.1.2000

2. MATERIALS AND METHODS

2.1. TEST SYSTEM/SUBJECTS

Experimental animals:	NMRI mice, HsdWin:NMRI.
Age/weight:	Group I: 9 weeks/40 ± 4 g (Mean ± S.D.) Group II: 6 weeks/34 ± 2 g (Mean ± S.D.)
Source:	Harlan, Netherlands
Number of animals in the study:	72
Number of animals/group:	8
Acclimatisation period:	5 weeks or 8 days
Principles for selection into test groups:	Animals were selected randomly by hand into different treatment groups.
Identification of animals:	The animals were marked on their tails with codes in different colors.
Grounds for selection of species:	Mice are commonly used in studies of this type.

2.2. ENVIRONMENTAL CONDITIONS

Animal care:	The animals were cared and checked daily by the experimenters and/or personnel of the Central Animal Laboratory. The bedding of the animals was changed twice and water bottles once a week.
Number of animals/cage:	5-8 mice/cage.
Cage Type:	Polycarbonate Macrolon III (Scanbur AS, Denmark).
Bedding:	Aspen chips (Tapvei Oy Kaavi, Finland). The results of the analysis for specified contaminants are attached (Appendix 3).
Water:	Community tap water, *ad libitum*, except during the experiments. The results of the analysis for specified contaminants are attached (Appendix 4.).
Fodder:	RM1 (E) SQC, Special Diet Service, Witham Essex, England. Certificate detailing nutritional composition and levels of specified contaminants is attached (Appendix 5.).

Ambient temperature:	21 ± 2.5 °C
Humidity:	50 % ± 15 %
Illumination:	12-h dark/light cycle; lights on from 7.00 to 19.00 and lights off from 19.00 to 7.00.
Room numbers:	Experimental Room 312, BioCity, C-department Colony Room 309, BioCity, C-department

2.3. REAGENTS

2.3.1. Test compounds

Hydroxymatairesinol (mw. 374)

Vehicle:	PEG 300 Sigma (Chemicals Co, St Louis, MO, USA)
Batch:	00799
Storage:	at 4 °C, desiccated, protected from direct light

2.3.2. Reference compounds

d-Amphetamine sulfate (mw. 368.5)

Manufacturer:	Sigma Chemicals Co, St Louis, MO, USA
Vehicle:	0.9% NaCl
Lot:	38F-0927
Storage:	RT

Medetomidine (mw. 200.28, Domitor 1 mg/ml,)

Manufacturer:	Orion Pharma, Espoo, Finland
Vehicle:	0.9 % NaCl (saline)
Lot:	ZH 31-3
Batch:	11/98
Storage:	at room temperature protected from direct light

2.3.3. Rationale for dose selection

In the experiments assessing the pharmacodynamic efficacy of HMR ,e.g. antitumor activity (Saarinen et al. Nutrition and cancer 2000 (36):207-216) a dose 15 mg/kg, (p.o.) have been found to be effective.

Thus, the doses selected for the present study (10, 30 and 100 mg/kg, p.o.) were within this therapeutic range or exceed that.

2.3.4. Preparation and handling of test compound solutions

Fresh test compound solutions were prepared on each experimental day. HMR was dissolved in polyethylene glycol (PEG) 300 and reference compound d-amphetamine was dissolved either in PEG or 0.9 % NaCl. Another reference compound medetomidine was

diluted from Domitor® solution with 0.9 % NaCl. HMR solutions were sonicated at 40 °C for 8-15 min. d-Amphetamine and medetomidine test solution were prepared once a week

2.4. EXPERIMENTS

2.4.1. Procedure

The spontaneous motor activity of the animals was measured in transparent polypropylene cages with transparent plastic lid by Photobeam Activity System (PAS, Cage Rack®, San Diego Instruments, San Diego, USA). Horizontal and vertical activities (rearing) were measured with photobeam frames located at the height of 3 cm and 6 cm from the bottom of the cage, respectively. Ambulatory, vertical and fine movement activity counts accumulated over measurement period were recorded with PC installed with PAS-software.

2.4.2. Administration of compounds

Vehicle (PEG 300), different doses of HMR or reference compound, d-amphetamine, were given p.o. (Experiment 1) or s.c. (Experiment 2) in volume of 10 ml/kg, while another reference compound, medetomidine, was given s.c. (in saline 10 ml/kg). Immediately after the treatments, the animals were placed in the activity measurement cages and their activities were recorded for 180 min at 30-min intervals.

Table 2.1. Treatments in Experiment 1

Groups	Treatment	Dose
I	Vehicle (PEG 300)	-
II	Medetomidine	30 µg/kg
III	Amphetamine	2.0 mg/kg
IV	HMR	10 mg/kg
V	HMR	30 mg/kg
VI	HMR	100 mg/kg

$n_i = 8$, $n = 48$

Purpose of the amphetamine-treated group in the original Study Plan was to serve as positive control group expressing drug-induced locomotor stimulation. d-Amphetamine was given orally similarly to the test compound. However, it was found that via oral route this dose of amphetamine does not induce locomotor stimulation (see section 5.2.1). In order to demonstrate stimulation, an additional experiment was performed (Experiment 2), and the same amphetamine dose was given subcutaneously. The effect of a largest HMR dose (100 mg/kg, p.o.) was tested as well.

Table 2.2. Treatments in Experiment 2

Group	Treatment	Dose
I	Vehicle	-
II	d-Amphetamine	2.0 mg/kg (s.c.)
III	HMR	100 mg/kg

2.4.3. Data collection

The data was collected with PAS software and transferred to MS-Excel worksheet. Ambulatory, vertical (rearing) and fine movement activity counts were used as an index of motor activity of the animals.

2.4.4. Statistics

Means, standard deviations and standard errors for each group were calculated. The data was first tested with analysis of variance for repeated measures (ANOVA) for all treatments (groups) and when this overall analysis showed significant difference, also between groups comparison were performed with ANOVA for repeated measures. In these analyses, Bonferroni adjustment was used in order to control type I error. When the data was not normally distributed, a logarithmic transformation was performed.

2.4.5. Termination of the experiments

At the end of the experiment, all animals were sacrificed with CO_2.

3. ARCHIVING

Study plan, final report and original data from different experiments are retained in the archive of PreFa (Tykistökatu 6B) at least for 10 years. After that, the further treatment of the documentation is decided together with the Sponsor. The documentation or parts of it may be delivered to the Sponsor on request before 10-year term. No data or documentation will be destroyed without a written permission from the Sponsor.

4. DEVIATIONS FROM STUDY PLAN

Due to lack of effect of amphetamine administered p.o., an additional experiment was conducted in which it was given s.c. This modification is described and approved by the Study Director and the Sponsor in the Amendment 1 to the Study Plan.

5. RESULTS

5.1. BODY WEIGHTS

Average (± S.D.) body weights of the animals in different treatment groups in the Experiments 1 and 2 are shown in table 5.1. There was no differences in the body weights of the animals between the groups in either experiment (Experiment 1: $F = 2.1$, $p = 0.085$ and Experiment 2: $F = 0.76$, $p = 0.48$, ANOVA).

Table 5.1. Average body weights (± S.D.) of the animals in different treatment groups in Experiment 1.

Experiment 1			**Body Weight (g)**			
Group	**Treatment**	**Dose**	**Mean**	**S.D.**	**Range**	n_i
I	Vehicle (PEG 300)	-	40	3	37-44	8
II	Medetomidine	30 µg/kg	40	4	35-46	8
III	Amphetamine	2.0 mg/kg	37	3	33-43	8
IV	HMR	10 mg/kg	42	4	36-48	8
V	HMR	30 mg/kg	39	3	35-46	8
VI	HMR	100 mg/kg	40	4	33-44	8

Experiment 2			**Body Weight (g)**			
Group	**Treatment**	**Dose**	**Mean**	**S.D.**	**Range**	n_i
I	Vehicle (PEG 300)	-	34	1	33-35	8
II	Amphetamine	2.0 mg/kg	34	2	31-36	8
III	HMR	100 mg/kg	35	2	33-37	8

5.2. EFFECTS OF HMR ON MOTOR ACTIVITY OF THE ANIMALS

5.2.1. Drug effects in Experiment 1

ANOVA showed significant differences in all measures of motor activity between the different treatment groups (fig 5.1). For ambulation, ANOVA showed significant group effect ($F = 4.2$, $p < 0.01$) and time x group interaction ($F = 4.9$, $p < 0.001$). In pairwise comparisons, only medetomidine group differed significantly from the control group (group x time interaction: $F = 22.7$, $p < 0.001$). Medetomidine clearly decreased motor activity during first 90 min of the test, but the activity these animals was slightly increased in the end of the measurement period relative tot the control group. Therefore the group effect did not reach statistical significance when the p-value (0.0072) was Bonferroni adjusted. The statistical analysis of other measures of motor activity, i.e. fine movements and rearing showed similar results in that only medetomidine-treated group differed significantly from the control group (See Appendix 2). Another reference compound, d-amphetamine, did not have significant effect.

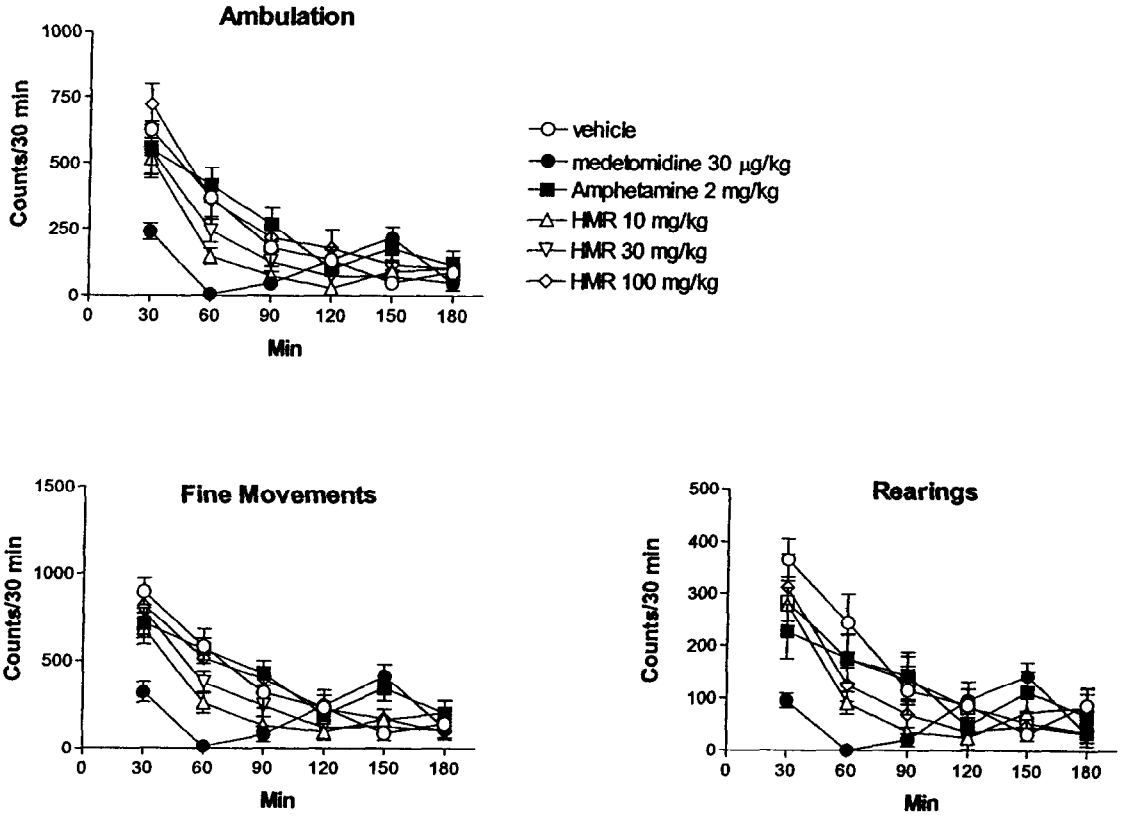

Figure 5.1. The effects of different doses of HMR and references compounds, medetomidine and amphetamine on ambulatory activity, fine movements and rearing activity in mice. The given are means ± S.E.M.

5.2.2. Drug effects in Experiment 2

ANOVA showed significant group effects for ambulation ($F = 8.9$, $p < 0.01$), and fine movements ($F = 9.8$, $p < 0.01$), but for rearing $F = 3.23$, $p = 0.06$). In pairwise comparisons, amphetamine-, but not HMR-treated group differed significantly from the control group. E.g., for ambulation, ANOVA conducted for vehicle and amphetamine-treated groups, showed a significant group effect ($F = 8.5$, $p < 0.05$) confirming significant effect of amphetamine. As in case of overall ANOVA, for rearing, there was no significant group effect ($F = 2.37$, $p = 0.15$), but significant treatment x group interaction ($F = 8.6$, $p < 0.001$). This was due to decrease of rearing activity by amphetamine during first 30-min period of the test, which was followed by stimulation in later phase (fig 5.2).

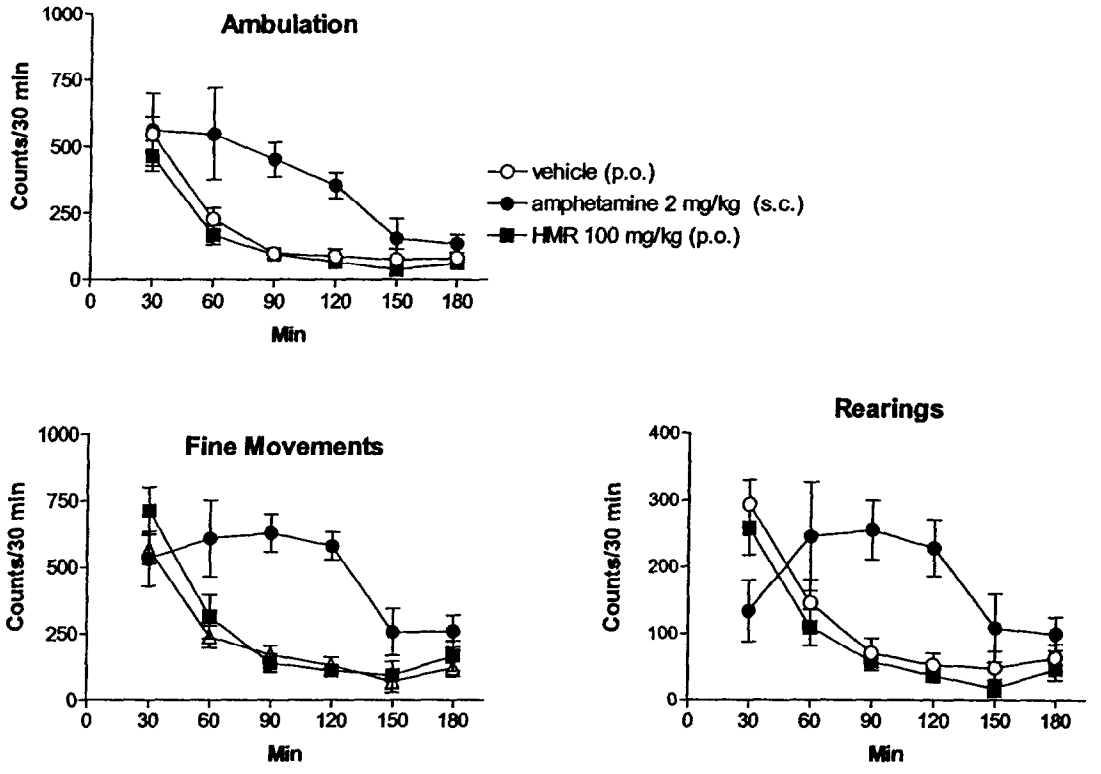

Figure 5.2. The effects of HMR (100 mg/kg, p.o.) and amphetamine (2 mg/kg, s.c.) on ambulatory activity, fine movements and rearing activity in mice. The given are means ± S.E.M.

6. CONCLUSIONS

These results demonstrate that HMR (10-100 mg/kg, p.o.) does not alter spontaneous motor activity of the NMRI mice.

7. DISTRIBUTION OF THE REPORT

The Report is written in duplicate, one original copy being retained in the Archives of PreFa and one delivered to the Sponsor.

Appendices

1. Values from the individual animals
2. Statistics
3. Report from analysis of bedding for contaminants
4. Report from analysis of water for contaminants
5. Report from analysis of fodder for nutritional composition and levels of specified contaminants.